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Immunological clearance of ^{75}Se-labelled *Trypanosoma brucei* in mice

II. MECHANISMS IN IMMUNE ANIMALS

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Summary. Using trypanosomes labelled with [^{75}Se]-methionine a series of experiments was conducted to investigate the respective roles of antibody, macrophage activation and complement in the removal of trypanosomes from the circulation of immune mice. It was found that clearance in such animals is largely accomplished by antibody-mediated hepatic phagocytosis, which, at least in passively immunized animals, is dependent on opsonization involving C3. No evidence was found to suggest that intravascular lysis or activated macrophages are involved in immune clearance.

INTRODUCTION

Although the development of immunity to trypanosomiasis in domestic animals is only occasionally a sequel to natural infection due to the parasites' ability to undergo repeated antigenic variation, protective immunity against a particular antigenic variant can be readily induced by a variety of vaccination procedures (see reviews by Terry, 1976; Clarkson, 1976; Murray & Urquhart, 1977; Holmes, 1980).

Such immunity is thought to depend largely on serum antibody. Thus humoral responses to specific antigenic variants have been demonstrated *in vitro* with a variety of methods including agglutination, trypanolysis, infectivity neutralization (see Lumsden, Herbert & McNeillage, 1973) and metabolic inhibition (Desowitz, 1956; Diggs, Flemings, Dillon, Snodgrass, Campbell & Esser, 1976).Passive transfer of protection has also been reported using immune serum (Seed & Gam, 1966) and recently Campbell & Phillips (1976) have shown that resistance can be transferred to syngeneic recipients with serum or B lymphocytes but not by T lymphocytes.

There is also evidence that macrophages may play an important role in protection against trypanosomes, particularly in the presence of homologous antiserum (Lumsden & Herbert, 1967; Goodwin, 1970), although a role for activated macrophages *per se* has also been proposed (Murray, Jennings, Murray & Urquhart, 1974; Corsini, Clayton, Askonas & Ogilvie, 1977; Stevens & Moulton, 1978).

Finally the role of complement in immune clearance is still unresolved. Hypocomplementaemia, attributed to complement activation by the parasites, occurs in trypanosome infections and has been suggested as a mechanism whereby the parasites may avoid complement-dependent trypanolysis (Nielsen & Sheppard,

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1977; Musoke & Barbet, 1977). However, Shirazi, Holman, Hudson, Klaus & Terry (1980) have concluded that IgM antibodies alone, acting as agglutinins, are probably adequate to control blood-stream infections.

The recent development of a labelling technique for trypanosomes utilizing [^{75}Se]-methionine (MacAskill, Holmes, Whitelaw, Jennings & Urquhart, 1978; Holmes, MacAskill, Whitelaw, Jennings & Urquhart, 1979) has now made possible *in vivo* studies on immunological clearance of trypanosomes. This paper reports the results of a series of such experiments using ^{75}Se-labelled *Trypanosma brucei* to investigate the respective roles of antibody, macrophage activation and complement in the removal of trypanosomes from the circulation of immune mice.

MATERIALS AND METHODS

Parasite

A strain of *T. brucei* derived from a stabilate of TREU 226 was used. Rats and mice which had been sub-lethally irradiated (Lumsden *et al.*, 1973) were infected with 1×10^5 and 1×10^4 organisms, respectively, by intraperitoneal (i.p.) injection. This trypanosome causes an acute and fatal infection of 5–7 days' duration in rats and mice.

Laboratory animals

Female Hooded Lister rats weighing between 130 and 180 g, female CFLP mice weighing 20–30 g and AKR strain mice genetically deficient in the fifth component of complement (C5) were used; prior to use the absence of C5 in AKR mice was confirmed by haemolytic radial diffusion (Lachmann & Hobart, 1978).

Radiolabelling technique

Radiolabelled trypanosomes were prepared using [^{75}Se]-methionine in an *in vivo* labelling method recently described by Holmes *et al.* (1979).

Trypanosomes were separated from infected blood using a DEAE cellulose (Whatman Chromedia DE52) column according to the method of Lanham & Godfrey (1970); the isolated trypanosomes were then washed three times in ice-cold phosphate-glucose buffered saline (PGBS) at pH 8·0 and enumerated using an improved Neubauer haemocytometer (Lumsden *et al.*, 1973). The radioactivity of labelled trypanosomes and all other radioactivity determinations were carried out on a Packard automatic gamma scintillation counter.

Injection of labelled trypanosomes and sampling

The suspension of washed ^{75}Se-labelled trypanosomes was diluted with PGBS to give an injection dose of 1×10^8 organisms (approximately 4000 c.p.m.) per mouse.

One hour after intravenous (i.v.) injection of labelled parasites, the mice were killed and the distribution of radiolabelled parasites in various organs determined as previously described (Holmes *et al.*, 1979).

The radioactivity of each organ and that of the calculated total blood volume was expressed as a percentage of the injected activity.

Immunization procedure

Groups of mice were immunized against *T. brucei* TREU 226 by i.p. injection of 1×10^4 trypanosomes and drug therapy 5 days later with diminazene aceturate (40 mg/kg, Berenil, Farbwerke, Hoechst). The immunized mice when subsequently challenged 4 weeks later with 1×10^4 organisms of the same stabilate were found to be totally resistant as judged by failure to detect parasites on examination of wet blood films over a period of 4 weeks.

Irradiation of mice

Mice were sub-lethally irradiated 1 day prior to infection with 650 rad in a ^{60}Co source.

Decomplementation of mice

Mice were decomplemented by three i.v. injections over 24 h of purified cobra venom factor (CVF) (total 25 μl/mouse). The trypanosome clearance ability of the animals was determined 24 h after the final injection of CVF. Serum complement (C3) levels of the mice were quantified by rocket electrophoresis using rabbit anti-mouse C3.

Stimulation of the mononuclear phagocytic system

Non-specific stimulation of the mononuclear phagocytic system (MPS) was achieved by i.v. injection of either *Mycobacterium bovis* (BCG Vaccine, Glaxo) $1{\cdot}5 \times 10^7$ organisms/mouse or *Corynebacterium parvum* (Coparvax, Wellcome) 1·4 mg/mouse, 10 days prior to clearance studies.

Preparation of hyperimmune serum

Hyperimmune serum (HIS) was obtained from rats by infection and drug cure with Berenil (40 mg/kg) on day

4 of infection. The rats then received two subsequent challenges of 1×10^5 organisms, i.p. 4 days and 24 days after drug therapy. The serum was then collected 9 days later and pooled.

In vitro *treatment of trypanosomes with serum*

(a) Neutralizing antibody infectivity tests were performed on sera (Lumsden *et al.*, 1973). The test well contained 200 μl serum, 50 μl guinea-pig serum and 5×10^4 trypanosomes/250 μl PGBS. After 30 min at 4°, the contents of the test well were taken up in a syringe and five normal mice were each inoculated i.p. with an equal aliquot. The mice were then monitored daily for the appearance of parasites by wet blood film examination.

(b) Agglutination titres of fresh hyperimmune serum were determined in microtitre plates. Duplicate test wells contained doubling dilutions of 25 μl of serum in PBS, to which was added 25 μl of a trypanosome suspension containing 5×10^7 organisms/ml. The plates were maintained at room temperature for 30 min and the agglutination titres then assessed on an inverted microscope.

(c) Trypanolytic activity of fresh hyperimmune serum was measured *in vitro* using ^{75}Se-labelled trypanosomes. The experiment was conducted in two parts. In the first duplicate doubling dilutions of 0·5 ml of hyperimmune or normal serum in PBS were prepared in glass vials and 0·2 ml of PBS or fresh undiluted guinea-pig serum added. To these were added 0·1 ml of a trypanosome suspension containing 5×10^7 organisms/ml. The tubes were incubated at 37° for 30 min, after which 0·2 ml PBS was added, the contents mixed thoroughly and centrifuged at 2500 *g* for 15 min. 0·5 ml of the supernatant was pipetted into a separate tube and the radioactivity determined of both the supernatant and the residue.

In the second part a broadly similar protocol was followed except that a single dilution (1:8) of HIS was selected and the tubes were incubated at 37° for 30, 60, 90 and 120 min.

The radioactivity of the supernatant of each tube was expressed as a percentage of the total count to obtain 'percentage lysis'.

(d) Clearance studies of labelled trypanosomes were conducted following their *in vitro* treatment with serum. This consisted of a modified infectivity neutralization test in which ^{75}Se-labelled trypanosomes were incubated at 4° for 30 min in HIS or in normal serum. The trypanosomes were then washed three times with PGBS.

Groups of mice were inoculated i.v. with a suspension containing 1×10^8 trypanosomes as described above.

Statistics

Variation around the mean is expressed as the standard error.

RESULTS

The effect of hyperimmune serum on clearance of labelled *T. brucei* in normal mice

Previous studies (Holmes *et al.*, 1979) showed that rapid removal of trypanosomes from the blood of actively immunized mice was achieved principally by the liver. The role of antibody in this process was investigated both *in vivo* and *in vitro*, using HIS prepared in rats. Firstly, the effect of HIS was assessed *in vivo* by injecting normal mice with labelled trypanosomes and 15 min later giving various i.v. doses of HIS. The results (Fig. 1) showed that a level of circulating antibody could be readily attained which facilitated hepatic uptake of trypanosomes to a degree similar to that of actively immunized mice.

Secondly, the effect of pre-treatment of labelled trypanosomes with HIS *in vitro* prior to injection into normal mice was investigated. The results presented in Table 1 clearly indicated that such treatment can induce levels of hepatic uptake which were similar to those in either actively or passively immunized mice.

Macrophage function in the absence of antibody

Although the previous experiment showed that unsensitized hepatic macrophages are as efficacious in the presence of passively acquired HIS as those in

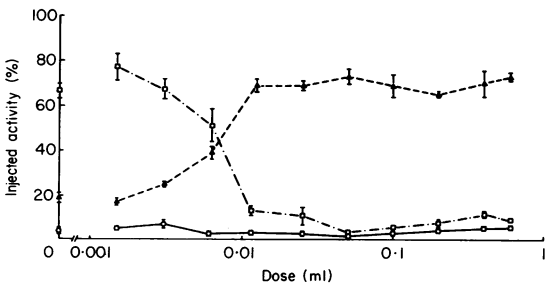

Figure 1. The tissue distribution of ^{75}Se-labelled trypanosomes in mice passively immunized with various doses of hyperimmune serum: (dashed line) liver; (dashed/dotted line) blood; (solid line) spleen.

Table 1. The effect of *in vitro* treatment with hyperimmune serum or normal serum on the tissue distribution of ^{75}Se-labelled trypanosomes in normal mice.

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
Hyperimmune serum	<3·0	59·4±1·8	<3·0
Normal serum	<3·0	9·5±0·5	69·2±5·6

actively immunized animals, it was thought useful to test the phagocytic efficiency of antigen-experienced macrophages in the absence of antibody. In this experiment groups of mice were immunosuppressed by sublethal irradiation with 650 rad: this level of irradiation suppresses antibody production but has been reported not to affect phagocytosis (Howard, 1970; Lumsden *et al.*, 1973). Subsequently, these mice and normal controls were infected. Three days later, the animals were cured with Berenil (40 mg/kg) and after a further 4 days injected with ^{75}Se-labelled trypanosomes.

The results presented in Table 2 again showed that the immunizing procedure of infection and treatment resulted in high hepatic clearance of trypanosomes. However, those mice which had received prior irradiation had markedly reduced clearance values to levels only slightly above those of normal animals.

There are two possible explanations for this result. Firstly, prior irradiation may have damaged phagocytic function and secondly, irradiation may have prevented the production of effective antibody levels.

The first possibility was examined in a group of twenty normal mice, some of which were irradiated with 650 rad and 8 days later injected with ^{75}Se-labelled trypanosomes followed 15 min later by 0·2 ml of 1:8 dilution of HIS. The results (Table 3) showed that prior irradiation had not impaired hepatic uptake of trypanosomes.

The second possibility, i.e. suppression of antibody production by prior irradiation was confirmed by infectivity neutralization tests. In such tests, pooled sera from irradiated, infected and treated mice were shown not to possess protective antibodies (100% mortality in five recipient mice) whereas sera of similar non-irradiated mice abolished the infectivity of trypanosomes to normal mice (100% survival in five recipient mice).

The effect of MPS activation on phagocytic removal of ^{75}Se-labelled trypanosomes

Since expansion and activation of the MPS is a characteristic feature of trypanosome-infected animals (*vide supra*) it is possible that, apart from antibody-induced clearance, trypanosomes may be also removed from the circulation in such animals by non-specific phagocytosis.

In order to test this hypothesis, the clearance of trypanosomes from the circulation was measured in normal mice which had previously received either BCG or *C. parvum* as non-specific MPS stimulants. Both have been shown to cause increases in liver and spleen weights associated with hyperactivity of their macrophages and increased phagocytosis of carbon particles and bacterial cells (Stiffel, Mouton & Biozzi, 1970).

The results in Table 4 showed that prior treatment with either BCG or *C. parvum* was associated with increases in hepatic and splenic weights. This was

Table 2. The effect of prior irradiation on the tissue distribution of ^{75}Se-labelled trypanosomes in actively immunized* and normal mice

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
Irradiated immunized	3·8±0·3	13·2±3·1	55·3±7·7
Non-irradiated immunized	<3·0	49·3±1·8	<3·0
Irradiated drug treated	4·7±1·7	8·3±0·6	67·6±8·9
Non-irradiated drug treated	4·5±0·3	9·2±0·3	73·9±5·7
Irradiated non-immunized	<3·0	8·3±0·5	61·7±6·0
Non-irradiated non-immunized	3·2±0·6	6·5±0·7	68·5±2·9

* Immunized by infection followed by drug therapy.

Table 3. Effect of prior irradiation on the tissue distribution of ^{75}Se-labelled trypanosomes in passively immunized* and normal mice

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
Irradiated immunized	<3·0	77·8±1·6	<3·0
Irradiated non-immunized	<3·0	14·3±1·2	80·6±5·9
Non-irradiated immunized	<3·0	67·4±2·4	<3·0
Non-irradiated non-immunized	<3·0	11·0±0·7	77·5±3·2

* Passively immunized by i.v. injection of 0·2 ml HIS 15 min after the injection of labelled trypanosomes.

Table 4. The effect of non-specific MPS stimulants on the tissue distribution of ^{75}Se-labelled trypanosomes

Treatment	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
BCG	5·5±0·5 (0·81±0·15)*	8·8±0·5 (6·99±0·12)	63·9±2·9
C. parvum	13·3±1·1 (2·25±0·11)	10·2±0·7 (13·15±0·37)	49·3±2·6
Non-treated	3·2±0·65 (0·37±0·02)	6·5±0·7 (6·27±0·21)	68·5±2·9

* Figures in parentheses are organ weights as percentage of total body weight.

reflected by proportionate increases in trypanosome uptake by both organs. However, the level of hepatic uptake was very much less than that obtained with HIS, i.e. <11% compared with approximately 60% (Fig. 1.).

The role of complement in immune clearance

The experiments so far described have clearly demonstrated that antibodies are essential in inducing blood clearance of trypanosomes. However, the results do not show whether whole opsonized organisms can be removed or if complement-mediated lysis is an essential prerequisite for phagocytosis. In order to investigate the latter possibility, two experiments were conducted. In the first, the trypanolytic and agglutinating activity of freshly collected HIS were determined *in vitro*. In the second, the *in vivo* clearance ability of passively immunized mice, which were either genetically deficient in C5 or C3-depleted by prior treatment with CVF, was measured. The former mice permit an investigation of the role of the terminal (lytic) pathway of complement activation whereas the latter should reveal the role of C3 in immune clearance.

Trypanolytic and agglutinating ability of HIS in vitro. Fresh HIS alone was found to have no significant trypanolytic activity above PBS control values (i.e. <10%) at various dilutions and for up to 2 h incubation. However, if guinea-pig serum was added 20–30% lysis occurred.

In comparison, HIS had pronounced trypanosome-agglutinating activity, i.e. a titre of 1/64.

Immune clearance in C5-deficient and C3-depleted mice. Immune clearance was measured in passively immunized mice by i.v. injection of ^{75}Se-labelled *T. brucei* followed 15 min later by an injection of 0·2 ml HIS (1:8 dilution). The results presented in Table 5 show that C5-deficient mice had hepatic uptake values only slightly lower than those of passively immunized normal mice. However, in passively immunized C3-depleted CFLP mice, in which C3 was reduced by 75%, the hepatic uptake remained at normal levels.

Table 5. The tissue distribution of ^{75}Se-labelled trypanosomes in passively immunized normal, C5-deficient and C3-depleted mice

Groups	Tissue distribution (% injected activity)		
	Spleen	Liver	Blood
C5-deficient immunized	<3·0	54·7±3·1	20·8±6·6
C5-deficient non-immunized	<3·0	11·1±1·3	73·7±5·6
C3-depleted immunized	8·7±0·9	10·1±0·8	58·3±3·2
Normal immunized	<3·0	67·4±2·5	<3·0
Normal non-immunized	6·4±1·1	11·0±0·7	77·5±3·2

DISCUSSION

The results presented in this paper and those reported previously (Holmes *et al.*, 1979) suggest that the ability of immune mice to remove trypanosomes from the circulation is largely dependent on phagocytosis by the liver. Thus, 60 min after the injection of radiolabelled trypanosomes into immune mice, approximately 60% of the injected activity was found in the liver and insignificant amounts, i.e. <3%, in the circulation; in

contrast, in control mice only about 8% of the activity was found in the liver and more than 60% in the circulation. The current results also indicate how this is achieved.

In theory the hepatic macrophages may remove trypanosomes: by phagocytosis of antibody-coated whole trypanosomes; by removal of fragments of trypanosomes subsequent to their immune lysis; through the action of macrophage cytophilic antibody; as a result of their previous exposure to trypanosome antigen; or non-specifically through the presence of an expanded and activated MPS.

From the results shown in Fig. 1 it is apparent that the amount of passively acquired circulating antibody is closely related to the degree of hepatic uptake and that blood clearance levels as high as those observed in actively immunized mice can be readily achieved. The fact that the activity retained by the organs examined does not completely account for all the injected activity suggests that some labelled trypanosomes are absent from the main systemic circulation. This may be either as a result of trapping in small capillaries (Goodwin, 1970) or by non-specific phagocytosis of low numbers of trypanosomes throughout the body.

The importance of antibody in producing high hepatic uptake was confirmed in the second experiment in which radiolabelled trypanosomes were exposed to HIS *in vitro*; on their subsequent injection into normal mice, a degree of hepatic uptake similar to that of actively or passively immunized mice was obtained. Furthermore, since the trypanosomes had been washed three times after incubation in HIS it is apparent that the antibody functioned as an opsonin rather than as a cytophilic antibody on the macrophages.

The importance of antibody and the relatively minor significance of macrophages activated by trypanosome infection or by immunostimulants was demonstrated in two studies in which macrophage activity in the absence of antibody was evaluated. In the first, suppression of antibody production by prior irradiation, at a level which did not impair phagocytic function, abolished the ability of infected and treated mice to remove high levels of radiolabelled parasites. In the second, MPS activation by the non-specific stimulants BCG and *C. parvum* only marginally increased clearance values above those of control animals and this was closely correlated with the degree of hepatomegaly and splenomegaly induced by these agents.

Finally, the possible role of complement-mediated lysis in immune clearance of trypanosomes was investgated using *in vitro* measurements of agglutinating and trypanolytic activity of HIS and the trypanosome clearance ability of passively immunized C5-deficient and C3-depleted mice. The results of all these experiments consistently indicated that complement-mediated lysis was neither a significant property of HIS *in vitro* nor a prerequisite to clearance *in vivo*. Thus no lytic activity of HIS could be demonstrated *in vitro* and deficiency of C5, which is essential in complement-mediated lysis, did not affect immune clearance. In contrast, a reduction in C3, which is necessary for full opsonic activity, prevented immune clearance in passively immunized mice.

These findings are in agreement with earlier *in vitro* studies on the phagocytic function of peritoneal macrophages for *T. gambiense* in which a close correlation was found between opsonising activity and agglutination titres of HIS (Takayanagi, Nakatake & Enriques, 1974). More recently, Ferrante & Jenkin (1978) have shown that *T. lewisi* maintained in diffusion chambers in the peritoneal cavity of immune rats were not lysed but strongly agglutinated at the end of the experiment.

It is also of interest that studies of *T. musculi* infections in normocomplementaemic, C5-deficient and C3-depleted mice showed that C3 depletion caused prolonged infections whilst C5 deficient mice responded to infection in a similar manner to normocomplementaemic mice (Jarvinen & Dalmasso, 1977). As a result of these findings they concluded that complement-mediated lysis was not involved in the control of *T. musculi*. Furthermore these workers suggested that a C3-dependent function such as phagocytosis may facilitate elimination of the parasites.

In conclusion, all our results show that the removal of *T. brucei* from the circulation of immunized mice is largely accomplished by antibody-mediated hepatic phagocytosis. This, at least in passively immunized mice, is dependent on opsonization involving C3. No evidence was found which might suggest that lysis is a prerequisite to macrophage uptake or that trypanosome-sensitized or non-specifically activated macrophages are involved in immune clearance.

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